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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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PROCESS FOR CELL CULTURING BY CONTINUOUS PERFUSION CULTURING OF CELLS

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PROCESS FOR CELL CULTURING BY CONTINUOUS PERFUSION CULTURING OF CELLS

The invention relates to a process for the culturing of cells by perfusion culturing of a cell culture comprising cell culture medium and cells, wherein cell culture medium is added to the cell culture and wherein the cell culture is circulated over a filter module comprising hollow fibers resulting in an outflow of liquid comprising less cells than the cell culture and wherein the flow within the filter module is an alternating tangential flow, wherein the cells are mammalian cells or yeast cells.

It has been found that by perfusion culturing of mammalian cells or yeast cells in this way, higher cell densities can be obtained. Furthermore, it was found that the process of the invention also leads to less cell aggregation. Cell aggregation is disadvantageous, because for example due to the heterogeneity in metabolic profiles of the cells within the cell aggregates, process control is more difficult.

This is surprising, because, although such a perfusion process is described in US 6,544,424 B1, US 6,544,424 only mentions that this process can be used for perfusion culturing of animal cells and nowhere discloses nor suggests that higher cell densities may be obtained if the perfusion process is applied to mammalian cells or yeast cells. Furthermore, although US 6,544,424 B1 discloses that the perfusion process diminishes aggregate formation of particulates and gelatin on the membrane surface of the hollow fibers, it does not disclose nor suggest that cells in the cell culture would aggregate less.

Perfusion culturing of cells has its conventional meaning in the art, i.e. it means that during culturing cells are retained by a separation device in which there is an outflow of liquid comprising less cells than prior to separation and in which there is an inflow of the cell culture medium.

Perfusion culturing includes, but is not limited to continuous flow and semi-continuous flow, for example step-wise flow or staggered flow.

With the term 'hollow fiber' is meant a tubular membrane. The internal diameter of the tube is preferably between 0.3 and 6.0 mm, more preferably between 0.5 and 3.0 mm, most preferably between 0.5 and 2.0 mm. Preferably, the mesh size in the membrane is chosen such that the size of the pores in the mesh is close to the diameter of the cells, ensuring a high retention of cells while cell debris can pass the filter. Preferably, the meshsize is between 3-30 µm.

Filter modules comprising hollow fibers are commercially available from for example Amersham.

With 'alternating tangential flow within the filter module' is meant that there is one flow in the same direction as (i.e. tangential to) the membrane surfaces of the hollow fibers, which flow is going back and forth and that there is another flow in a direction substantially perpendicular to said filter surface. Tangential flow can be achieved according to methods known to the person skilled in the art. For example, in US 6,544,424 it is described that alternating tangential flow can be achieved using one pump to circulate the cell culture over a filter module comprising hollow fibers and another pump to remove the liquid comprising less cells than prior to the filter separation.

In the process of the invention, any type of cell culture medium suitable for the culturing of cells can in principle be used. Guidelines for choosing a cell culture medium and cell culture conditions are well known and are for instance provided in Chapter 8 and 9 of Freshney, R. I. Culture of animal cells (a manual of basic techniques), 4th edition 2000, Wiley-Liss and in Doyle, A., Griffiths, J. B., Newell, D. G. Cell &Tissue culture: Laboratory Procedures 1993, John Wiley & Sons.

Generally, a cell culture medium for mammalian cells comprises salts, amino acids, vitamins, lipids, detergents, buffers, growth factors, hormones, cytokines, trace elements and carbohydrates. Examples of salts include magnesium salts, for example $MgCl.6H_2O$, $MgSO_4$ and $MgSO_4.7H_2O$ iron salts, for example $FeSO_4.7H_2O$, potassium salts, for example KH_2PO_4, KCl; sodium salts, for example NaH_2PO_4, Na_2HPO_4 and calcium salts, for example $CaCl_2.2H_2O$. Examples of amino acids are all 20 known proteinogenic amino acids, for example hystidine, glutamine, threonine, serine, methionine. Examples of vitamins include: ascorbate, biotin, choline.Cl, myo-inositol, D-panthothenate, riboflavin. Examples of lipids include: fatty acids, for example linoleic acid and oleic acid; soy peptone and ethanol amine. Examples of detergents include Tween 80 and Pluronic F68. An example of a buffer is HEPES. Examples of growth factors/hormones/cytokines include IGF, hydrocortisone and (recombinant) insulin. Examples of trace elements are known to the person skilled in the art and include Zn, Mg and Se. Examples of carbohydrates include glucose, fructose, galactose and pyruvate.

The pH, temperature, dissolved oxygen concentration and osmolarity of the cell culture medium are in principle not critical and depend on the type of cell chosen. Preferably, the pH, temperature, dissolved oxygen concentration and

osmolarity are chosen such that it is optimal for the growth and productivity of the cells. The person skilled in the art knows how to find the optimal pH, temperature, dissolved oxygen concentration and osmolarity for the perfusion culturing. Usually, the optimal pH is between 6.6 and 7.6, the optimal temperature between 30 and 39°C, the optimal osmolarity between 260 and 400mOsm/kg.

Examples of mammalian cells include: CHO (Chinese Hamster Ovary) cells, hybridomas, BHK (Baby Hamster Kidney) cells, myeloma cells, human cells, for example HEK-293 cells, human lymphoblastoid cells, PER.C6™ cells, mouse cells, for example NS0. Examples of yeast cells include *Saccharomyces cerevisiae, Phaffia rhodozyma, Pichia pastoris, or yeast cells from the genus* Hansenula. Preferably, mammalian cells are used, more preferably CHO, NS0, PER.C6™ cells, even more preferably PER.C6™ cells.

The rate of addition of cell culture medium (the inflow rate) is in principle not critical. In one embodiment of the invention, the cell culture medium is added at an inflow rate according to the following formula 1:

Inflow rate = SIR*total cell culture volume*viable cell density/10^6 (1)

, wherein the inflow rate is expressed in liters per day, wherein the SIR is the specific inflow rate, i.e. the rate in which the cell culture medium is fed to the cell culture expressed in nl per viable cell per day and wherein the viable cell density is the number of viable cells expressed in 10^6 cells/ml. The rate of addition of the cell culture medium influences the viability and the density of the cells.

The number of viable cells can be determined by the person skilled in the art, for example the trypan blue exclusion method. The specific inflow rate is preferably chosen between 0.01 and 0.3 nl/cell/day, more preferably between 0.01 and 0.2 nl/cell/day.

It may be of advantage to control the inflow rate using other factors, for example by using the level of glucose or oxygen as variables. For example, for PerC6 the glucose inflow rate is preferably chosen between 3 and 20 mmoles/l, more preferably between 5 and 15mmoles/l.

A person skilled in the art knows how to determine the outflow rate. The outflow rate of the liquid is determined by the inflow rate and is generally chosen at an equal rate.

In another embodiment of the invention, biomass (i.e. cells in cell culture) is removed at least once from the cell culture and additional cell culture

medium is added to the cell culture. Biomass removal may lead to higher cell densities. Biomass may be removed continuously or step-wise.

In the step-wise approach, biomass is removed continuously for a defined time period. If a step-wise approach is used, biomass removal is preferably started after the cells have reached a steady state. Biomass removal is preferably stopped after the cells have reached a new steady state.

If a step-wise approach is used, preferably between 2 and 40 % of the working volume per day, more preferably between 5 and 30% of the working volume per day, even more preferably between 10 and 25% of the working volume per day of the biomass is removed per biomass removal step. With 'working volume' is meant the total volume of the cell culture.

With biomass removal step is meant the time from the start to the stop of the biomass removal. Preferably, at least two biomass removal steps are employed. If more than one biomass removal step is employed, preferably, the amount of biomass removed in a later biomass removal step is less than in a former biomass removal step.

If a continuous approach is used, the biomass is removed continuously until the end of the cell culturing. Preferably, the continuous removal of biomass is started after the cells have reached a steady state. Preferably, biomass is removed at between 2 and 40 % of the working volume per day, more preferably between 3 and 30% of the working volume per day, even more preferably between 4 and 15% of the working volume per day.

The addition of the additional cell culture medium is used to compensate for the biomass removal. The feed wherein additional cell culture medium is added to the cell culture may be merged into the inflow feed, but may also be added in a separate feed. The person skilled in the art is aware how much additional cell culture medium is needed to compensate for the biomass removal. Generally, the rate of addition of the additional cell culture medium to the cell culture will be the same as the biomass removal rate.

In yet another embodiment of the invention, a biological substance is produced by the cells. The biological substances that can suitably be produced in the perfusion culturing by the cell are in principle all biological substances that can be produced by mammalian and yeast cells, for example therapeutic and diagnostic proteins, for example monoclonal antibodies, growth factors and enzymes, DNAs, which for example might be used in gene therapy, vaccines, hormones etc.

In the perfusion culturing process of the invention, the outflow of

liquid will comprise less cells and also more of the biological substance than prior to separation.

Preferably, the process according to the invention is used for the production of a biopharmaceutical product, which is a biological substance with a medical application. Examples of biopharmaceutical products are as follows (with examples of brand names of the corresponding biopharmaceutical product between brackets): Tenecteplase (TN Kase™), (recombinant) antihemophilic factor (ReFacto™), lymphoblastoid Interferon α-n1 (Wellferon™), (recombinant) Coagulation factor (NovoSeven™), Etanercept, (Enbrel™), Trastuzumab (Herceptin™), Infliximab (Remicade™), Basiliximab (Simulect™), Daclizumab (Zenapaz™), (recombinant) Coagulation factor IX (Benefix™), erythropoietin alpha (Epogen®), G-CSF (Neupogen®Filgrastim), Interferon alpha-2b (Infergen®), recombinant insulin (Humulin®), Interferon beta 1a (Avonex®), Factor VIII (KoGENate®), Glucocerebrosidase (Cerezyme™), Interferon beta 1b (Betaseron®), TNF alpha receptor (Enbrel®), Follicle stimulating hormone (Gonal-F®), Mab abcixmab (Synagis®, ReoPro®), Mab ritiximab (Rituxan®), tissue plasminogen activator (Activase ®, Actilyase®), human growth hormone (Protropin®, Norditropin®, GenoTropin™). Examples of DNAs with a possible medical application are gene therapeutic plasmid DNAs. Some gene therapeutic DNAs are presently tested in clinical trials for their medical application. Examples of vaccines are live, oral, tetravalent Rotavirus vaccine (RotaShield™), rabies vaccine (RanAvert™), Hepatitis B vaccin (RECOMBIVAX HB®, Engerix®) and inactivated hepatitis A vaccine (VAQTA™).

The biological substance in the outflow may be further purified in so-called downstream processing. Downstream processing usually comprises several purification steps in varying combinations and order. Examples of purification steps in the downstream processing are separation steps (e.g. by affinity chromatography and/or ion exchange chromatography), steps for the concentration of the biological substance (e.g. by ultrafiltration or diafiltration), steps to exchange buffers and/or steps to remove or inactivate viruses (e.g. by virusfiltration, pH shift or solvent detergent treatment).

The invention will now be elucidated by way of the following examples, without however being limited thereto.

Examples

Example: Determination of the number of viable cells:

The number of viable cells was determined as follows: An amount of cells stained with trypan blue was transferred to a fuchs Rosenthal haemacytometer. The chamber of the haemacytometer was placed under a microscope and an appropriate number of boxes was counted. The viable cell density was calculated using the following formula:

$$\text{Viable cell density } (X10^5 \text{ cells/ml}) = (A+B) \times {}^{E}/_{320} \qquad (2)$$

Wherein
A = number of non-stained cells in square A
B = number of non-stained cells in square B
E = dilution factor

On the Cell Culture Engineering conference of 8-12 March in Cancun, Mexico the following poster is presented:

Process Optimisation of the Human Cell Line PER.C^{tm} for the production of Biopharmaceticals
John Crowley, Maike Wubben, Edith Olthof, Johanne Coté, Rodney Gagne, José M. Coco Martin. DSM Biologics, Research & Development, P.O Box 454, 9700 AL Groningen, Netherlands.

Introduction

A number of expression platforms now exist for the production of biopharmaceuticals. Most of the new products must choose a mammalian system due in main part to the glycosylation machinery which these cells contain and others lack. However to date the cell mass and resulting productivity of these cells is a factor of 10 –100 times less than a corresponding microbial system if these cells had the machinery to make such products.

The PER.C6^{TM} cell line is a human cell line that possess a number of features that make it favourable for the production of biopharmaceuticals. In this study, the development of the three main modes of operation (batch, fed-batch and

continuous perfusion) for the PER.C6™ cell line were developed. The fed-batch process is based on the stoichiometric feeding of a balanced nutrient concentrate matched to the metabolic requirements of a model antibody producing PER.C6™ cell line.

A perfusion setup involves the separation of various components of the fermenter broth so that cells are retained, harvest is captured and medium refreshment occurs. The performance of a spinfilter, an acoustic device and an alternating tangential flow filter (ATF) within a developed high cell density continuous perfusion, the PER.C6™ cell line was assessed.

Materials & Methods

Cell line and maintenance : A PER.C6™ cell line was used in this study that produces a human IgG. Cells were maintained in a serum free commercial medium.The PER.C6™ cell line is a human embryonic cel-line immortalised with adenovirus type-5 (ad5) E1 gene using a phosphoglyceratekinase promoter.

Bioreactor Set-up: A 4L working volume reactor (Applikon, Netherlands) was used during this study. A Braun DCU3 controller (B.Braun, Germany) was used to operate the process at defined setpoints. Temperature was maintained at 37oC by a hot/cold finger. Dissolved oxygen concentration was controlled at 50% of air saturation by automatic adjustment of inlet gas composition through the headspace and intermittent sparging through a microporous sparger.

Cell Retention: Cells were retained in the reactor using two different devices. A Biosep 10L acoustic filter chamber and associated ASP991 controller (Applisens, Netherlands, a spinfilter basket (10um pore size) and an alternating tangential flow filter (ATF) were assessed. To maintain a constant culture volume a level sensor control loop was in operation.

Analytical methods : A cell count from the bioreactor was performed using the trypan blue exclusion method. Antibody concentration was determined by a analytical protein A column using a HPLC.

Results

Batch and Fed-Batch Cultures

Results obtained with the above materials and methods are shown in the figures.

Legends to the figures:

Figure 1: IgG concentration versus culture time (days) for a standard batch and developed fed-batch performed with the PER.C6™ cell line.

Figure 2: Antibody concentration versus culture time (days) for five 7L and one 70L batch fermentation performed with the PER.C6™ cell line.

Figure 3: Comparison of PER.C6™ cells in batch culture and a culture with a daily medium exchange.

Figure 4: Viable cell density ($x10^6$ cells/ml) versus culture time (days) for two different continuous perfusion fermentations of a Mab producing PER.C6™ clone differing specific perfusion rates (nl/cell/day) using a spinfilter separation device.

Figure 5: Growth of PER.C6™ cells in a continuous perfusion system with an acoustic device as a cell retention system.

Figure 6: Growth of PER.C6™ cells in a continuous perfusion system with an Alternating Tangential Flow filter as a cell retention system.

Figure 7: Productivity versus culture time (days) for two different continuous perfusion fermentations of a Mab producing PER.C6™ clone differing specific perfusion rates (nl/cell/day; closed circle ; 0.4 nl/cell/day) using a spinfilter separation device.

Figure 8: Productivity of PER.C6™ cells in a continuous perfusion system with an acoustic device as a cell retention system

Figure 9: Productivityof PER.C6™ cells in a continuous perfusion system with an Alternating Tangential Flow (ATF) filter as a cell retention system.

Figure 10: Photograph of a cell density and supernatant produced using PER.C6™ cells.

Figure 11: Cell density (x10^6 cells/ml) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using a spin basket separation device.

Figure 12: Volumteric production rate (g/L/day) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using a spin basket separation device.

Figure 13: Cell density (x10^6 cells/ml) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using an acoustic retention separation device.

Figure 14: Volumteric production rate (g/L/day) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using an acoustic retention separation device.

Figure 15: Viable cell density (x10^6 cells/ml) versus culture time (days) for PER.C6™ cells grown in a continuous perfusioin system using an alternating tangential flow (ATF) filtration device.

Figure 16: Volumetric production rate (g/L/day) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using an alternating tangential flow (ATF) filtration device.

Figure 17: Culture time (days) versus flow (L/day) and specific perfusion rate (SPR in nl/cell/day) for PER.C6™ cells cultured using a perfusion process.

Figure 18: Viable cell density (Figure 18: Culture time (days) versus flow (L/day and specific perfusion rate (nl/cell/day) for PER.C6™ cells cultured using a perfusion process.

Summary

Table 1:

Process	Max. Viable Cell Density (10^6 cells/mL)	Productivity	Yield Improvement Factor[1]
Batch	8-10	0.5 g/L	1
Fed-Batch	8-10	1.2 g/L	2.4
Continuous Perfusion			
Process 1	20-30	0.1-0.2 g/L/day	2.8-5.6
Process 2	20	0.6 g/L/day	16.8
Process 3	100	0.9 g/L/day	25.2

1 Normalized to a 14 day fed-batch process. Batch process is set as 1.

Conclusions:

- The initial performance of Per.C6™ in a fed-batch mode is already equivalent to other cell lines (1.2g/L).
- Early continuous perfusion experiments shows significant potential to achieve very high densities and product concentrations ($100x10^6$ cells/mL and 0.9 g/L/day).

Also on the same conference the following abstract is published:

Process optimization of the human cell line PER.C6™ for the production of biopharmaceuticals

John Crowley, Maike Wubben, Edith Olthof, Jose M. Coco Martin.

DSM Biologics, Research & Development, P.O Box 424, 9700 AL Groningen, Netherlands.

A number of expression platforms now exist for the production of biopharmaceuticals. The PER.C6™ cell line is a human cell line that possess a number

of features that make it favourable for the production of biopharmaceuticals. The factors involved in choosing such a platform will be discussed.

Having chosen an expression platform, the three main methods of production typically batch, fed-batch and continuous perfusion were developed for a model antibody producing PER.C6™ cell. The mode of operation is typically chosen on the basis of product concentration, quality and stability, scalability, simplicity, time and cost. In this presentation, the development of all three operation modes for the PER.C6™ cell line will be presented. The fed-batch process is based on the stoichiometric feeding of a balanced nutrient concentrate matched to the metabolic requirements of a model antibody producing PER.C6™ cell yielding equivalent productivity to other cell lines in a short time period.

A perfusion setup involves the separation of various components of the fermenter broth so that cells are retained, harvest is captured and medium refreshment occurs. The performance of a spinfilter, alternating tangential flow (ATF) and acoustic separation devices within a high cell density process using the PER.C6™ cell line will be presented. In conclusion, the early development of a continuous perfusion process for an antibody producing PER.C6™ cell will be presented yielding significantly high cell densities and volumetric production rates.

Process Example 2

This example relates to a process for culturing of PER.C6® cells by perfusion as defined previously.

Equipment: B.Braun fermenter control unit (Braun, Germany), 7L Braun vessel and headplate with associated pH, dissolved oxygen (DO) and level sensor probes (Braun, Germany), ATF-4 control unit and housing with associate hollow fibre membrane module (Refine Technology, USA).

Filter

Filter model: CFP-2-E-8SIP
Type: 0.2 micron
Area: 4600cm^2
Amersham Bioscience

Working volume

Setpoint: 4.1 L

Range: 3.8 – 4.7 L

ATF settings

Parameter	Setpoint	Range
Pressure rising setpoint (psi)	Variable	2 - 4
Pressure rising flow (L/min)	3.2	2.5 – 4.0
Exhaust flow (L/min)	3.2	2.5 – 4.0
Exhaust time (s)	Variable	3 - 8
Pre-pressure (psi)	Variable	5 - 9

Bleed rate

No biomass removal was applied to this process.

Additions

At day 16 two drops of antifoam (BC Antifoam Simethicone C100F; Basildon Chemicals Company Limited, USA), were added to the fermenteer in order to decrease the foam layer.

Materials: 6 mmole L-glutamine (Gibco) in VPRO mammalian medium (JRH, USA), 12% Na_2CO_3 is used to control the pH.

Cell Lines and Culture Conditions

A PER.C6™ cell line expressing a model IgG was investigated in this study. The PER.C6™ cell line is generated from retina-derived primary human cells. The PER.C6™ cell line is able to generate complete human monoclonal antibodies (including the glycans) (ref 1, ref 2).

Cells were cultured in shaking Erlenymer flasks at 110 rpm and 36.5°C . The headspace of these flasks was controlled using a mixture of 5% CO_2/Air.

Ref 1: Jones, D. H., van Berkel, P. H. C., Logtenberg, T. and Bout, A., 2002, 'PER.C6 cell line for human antibody production.', Gen. 22, ed. May 15.

Ref 2: Jones, D. et al., 2003, 'High-level expression of recombinant IgG in the human

cell line PER.C6.', Biotechnol. Prog. 19, 163-168.

Operation of Fermenter

Cells were cultured in a fermenter where dissolved oxygen tension, pH, temperature and agitation rate were controlled as detailed below.

Parameter	Setpoint	Range	
Temperature	36.5°C	35.5 – 37.5	
pH	> 6.7	7.5 –6.7, Active pH control using 12% Na_2CO_3 if pH<6.7	
DO	50%	40 –60%	
Agitation	100 – 300	Staged increase as viable cell density (VCD) increases;	
		VCD ($x10^6$ cells/ml)	Agitation (rpm)
		0.3 – 10	120
		10 –30	150
		30 –50	170
		50 –80	200
		80 – 100	230
		100 –120	260
		>120	300

Process Description : Cells are innoculated in a fermenter with an innoculation viable cell density range of 0.2 – 0.5 x 10^6cells/ml and a setpoint of 0.3 x 10^6 cells/ml. Perfusion is begun when the viable cell density > 2 x 10^6 cells /ml or at day 5 of the culture whichever is achieved first.

The perfusion rate is dependent on the cell density of the culture and the rates used are described in the table below. Both the flow rate and the dilution rate are adjusted as the cell density in the fermenter increases.

Perfusion rates utilized for culture of PER.C6™ cells

Viable cell density (x10⁶ cells/ml)	Specific perfusion rate (ml/cell.day)	Setpoint of specific perfusion rate (ml/cell.day)
Day 1 of perfusion	0.15 – 0.25	0.2
3 – 50	0.03 – 0.06	0.04
50 –80	0.025 – 0.035	0.03
>80	0.01 – 0.03	0.02

The actual data from this example (amongst others flowratres and specific perfusion rates used in this example) are shown in Table A below

Table A. Actual data from process example 2

Time	Flow rate (FR)	Dilution rate (D)	Specific perfusion rate (SPR)	Viable cell conc. (Vc)	Viability	product concentration	Specific production rate of IgG	Volumetric production rate
day	L/day	Working volume/day	nL/cell/day	10^6/mL	%	g/L	pg/cell/day	g/L-day
0	0.00	0.00	0.00	0.6	90	0.012	NA	NA
1	0.00	0.00	0.00	0.3	77	0.008	NA	0.000
2	0.00	0.00	0.00	0.3	73	0.008	0.0	0.000
3	0.00	0.00	0.00	0.5	80	0.013	12.1	0.000
4	0.00	0.00	0.00	0.9	87	0.019	9.3	0.000
5	0.00	0.00	0.00	1.4	92	0.033	12.0	0.000
6	2.39	0.52	0.20	2.6	95	0.035	5.5	0.009
7	1.06	0.24	0.05	4.9	95	0.054	9.5	0.017
8	2.70	0.57	0.08	7.3	97	0.073	7.2	0.026
9	2.60	0.57	0.05	12.3	97	0.067	3.5	0.040
10	4.29	0.95	0.05	18.6	97	0.115	7.8	0.069
11	5.40	1.20	0.04	26.9	97	0.140	7.0	0.137
12	6.80	1.48	0.05	31.8	96	0.127	5.6	0.179
13	7.39	1.68	0.04	41.4	99	0.129	5.6	0.202
14	8.28	1.88	0.04	44.3	98	0.139	5.8	0.238
15	10.26	2.33	0.03	68.3	98	0.116	4.4	0.269
16	10.70	2.43	0.03	86.1	99	0.151	4.6	0.318
17	12.10	2.63	0.03	80.3	98	0.163	4.9	0.397
18	11.83	2.57	0.02	112.3	98	0.292	7.6	0.592
19	12.50	2.78	0.02	123.0	99	0.291	6.6	0.780
20	12.09	2.57	0.02	126.0	99	0.293	6.3	0.781
21	11.91	2.59	0.02	135.0	98	0.332	6.5	0.806
22	13.70	2.98	0.02	127.5	97	0.395	8.2	1.012
23	10.00	2.17	0.02	128.5	95	0.470	9.3	1.114

Figure 17 shows the actual flowrates and specific perfusion rates used in process example 2.

Figure 18 shows the cell density achieved using the procedure described in this process example 2.

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CLAIMS

1. Process for the culturing of cells by continuous perfusion culturing of a cell culture comprising cell culture medium and cells, wherein cell culture medium is added to the cell culture and wherein the cell culture is circulated over a filter module comprising hollow fibers resulting in an outflow of liquid comprising less cells than the cell culture and wherein the flow within the filter module is an alternating tangential flow and wherein the cells are mammalian cells or yeast cells.
2. Process according to claim 1, wherein the alternating tangential flow is achieved using one pump to circulate the cell culture over a filter module comprising hollow fibers and using another pump to remove the liquid comprising less cells than the cell culture.
3. Process according to claim 1 or claim 2, wherein the cells are mammalian cells.
4. Process according to claim 3, wherein the mammalian cells are PER.C6™ cells.
5. Process according to any one of claims 1-4, wherein the cell culture medium is added at an inflow rate calculated according to formula 1:

   Inflow rate = SIR*total cell culture volume*viable cell density/10^6 (1)

   , wherein the inflow rate is expressed in liters per day, wherein the SIR is the specific inflow rate, i.e. the rate in which the cell culture medium is fed to the cell culture expressed in ml per viable cell per day and wherein the viable cell density is the number of viable cells expressed in 10^5 cells/ml.
6. Process according to claim 5, wherein the specific inflow rate is between 0.01 and 0.3 ml/cell/day.
7. Process according to any one of claims 1-6, wherein biomass is removed at least once from the cell culture and wherein additional cell culture medium is added to the cell culture.
8. Process according to claim 7, wherein the biomass removal is started after the cell have reached a steady state.
9. Process according to claim 7 or claim 8, wherein the rate of biomass removal is between 2 and 40% of the total volume of the cell culture.
10. Process according to any one of claims 1-9, wherein the cells produce a biological substance.

11. Process according to claim 10, wherein the biological substance is a monoclonal antibody.

12. Process according to claim 10 or claim 11, wherein the biological substance is further purified in downstream processing.

ABSTRACT

The invention relates to a process for the culturing of cells by continuous perfusion culturing of a cell culture comprising cell culture medium and cells, wherein cell culture medium is added to the cell culture and wherein the cell culture is circulated over a filter module comprising hollow fibers resulting in an outflow of liquid comprising less cells than the cell culture and wherein the flow within the filter module is an alternating tangential flow and wherein the cells are mammalian cells or yeast cells. The invention also relates to such a process wherein a biological substance, preferably an antibody is produced by the cells, which biological substance may be further purified in downstream processing.

Fig. 1

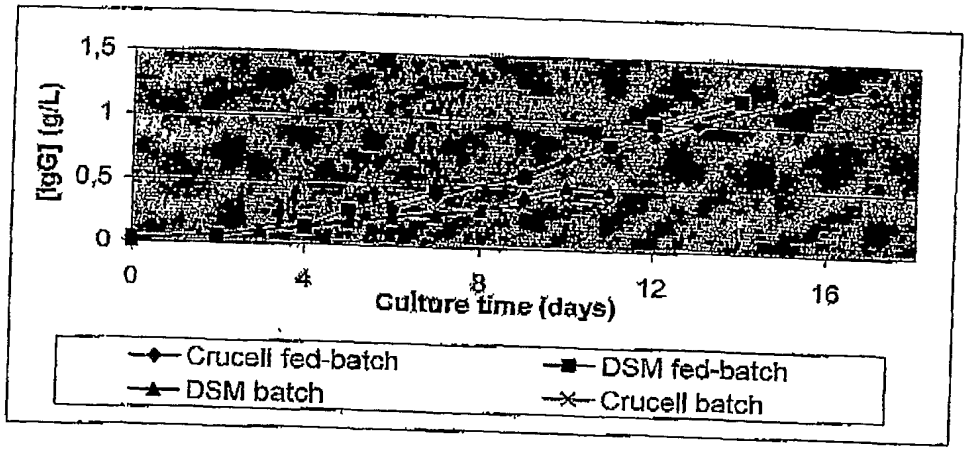

1,5
1
0,5
0
[IgG] (g/L)
0
4
8
12
16
Culture time (days)
Crucell fed-batch
DSM fed-batch
DSM batch
Crucell batch

Fig. 2

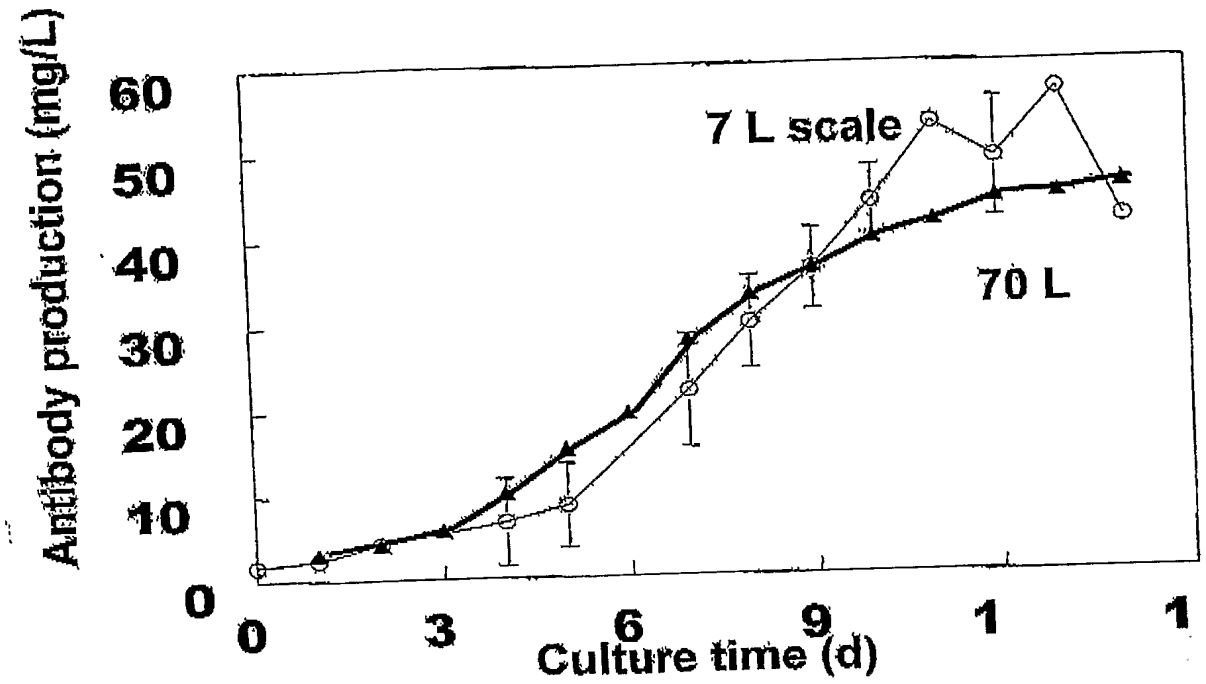

Antibody production (mg/L)
60
50
40
30
20
10
0
7 L scale
70 L
0
3
6
9
1
1
Culture time (d)

Fig. 3

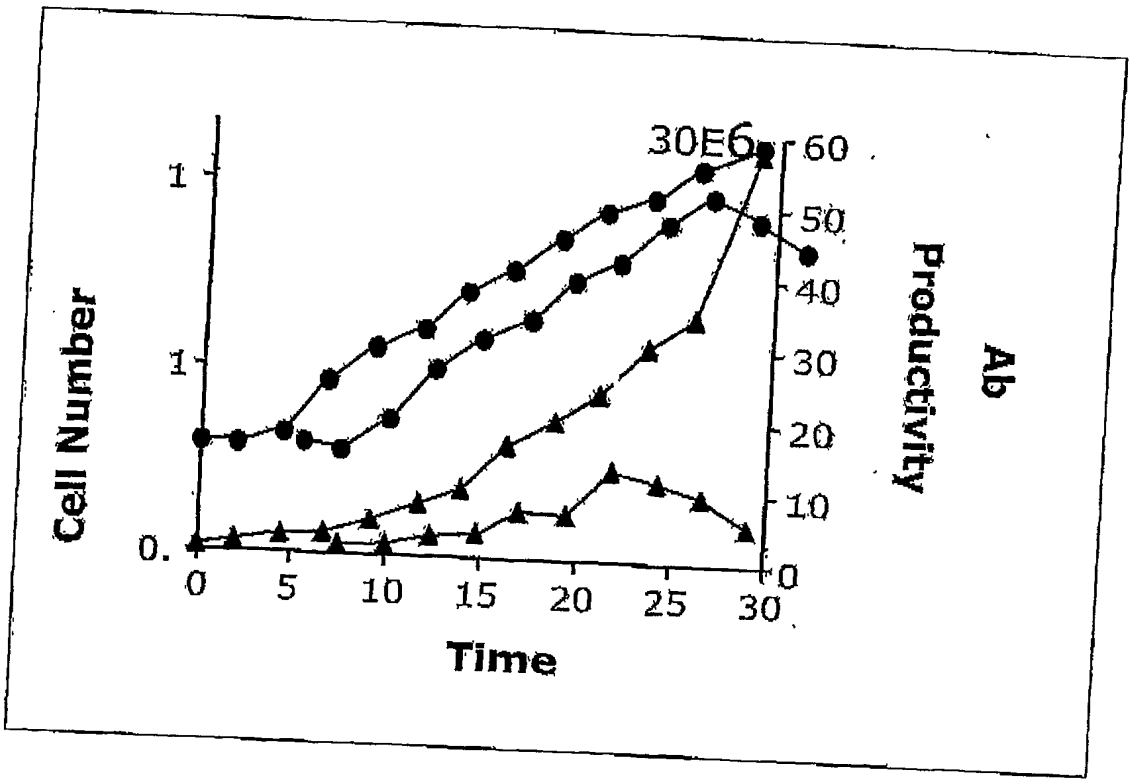

30E6
60
50
40
30
20
10
0
1
1
0.
0
5
10
15
20
25
30
Time
Cell Number
Ab
Productivity

Fig. 4

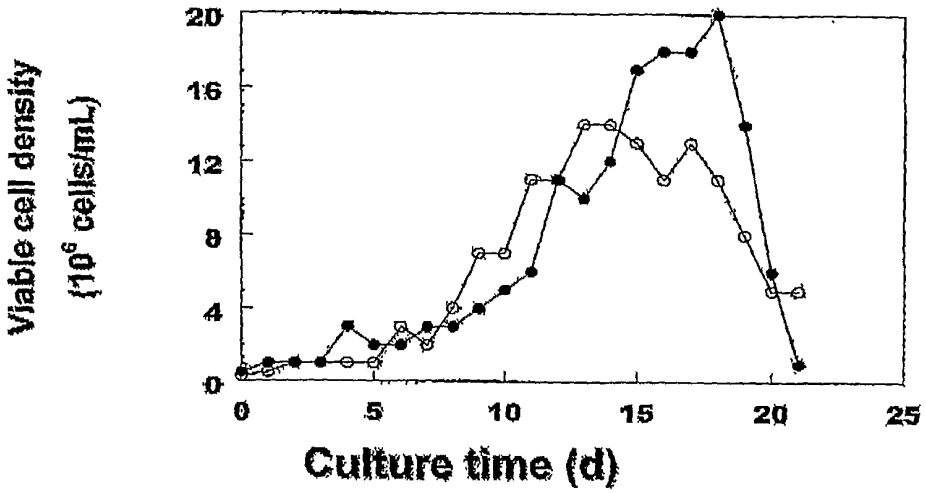

Viable cell density
(10^6 cells/mL)
20
16
12
8
4
0
0
5
10
15
20
25
Culture time (d)

Fig. 5

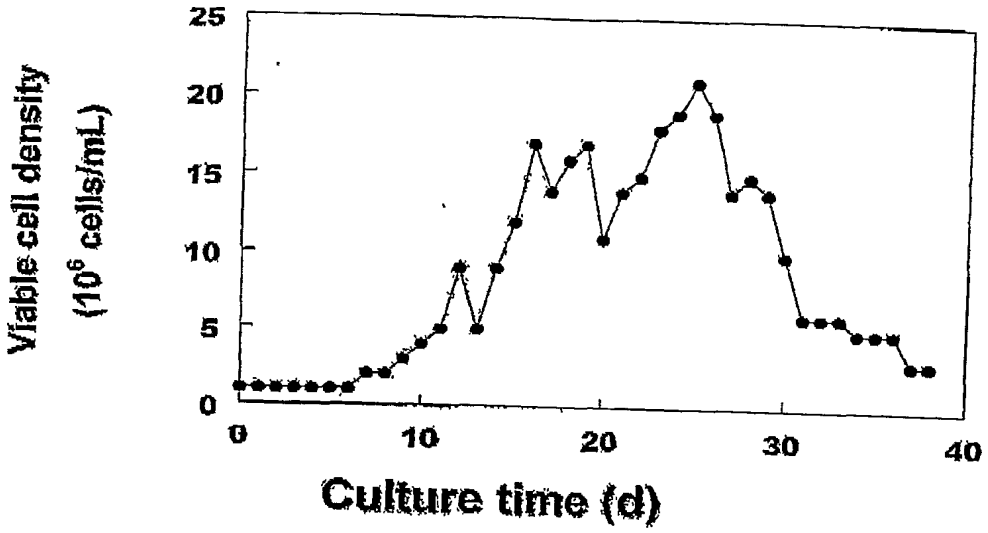

Viable cell density
(10^6 cells/mL)
25
20
15
10
5
0
0
10
20
30
40
Culture time (d)

Fig. 6

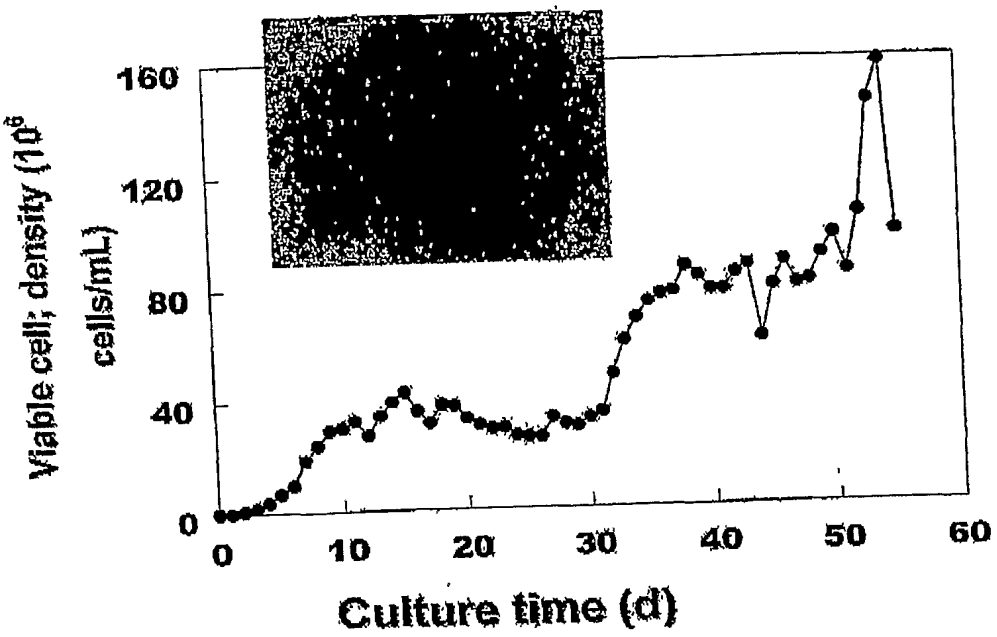

Viable cell; density (10^6 cells/mL)
160
120
80
40
0
0
10
20
30
40
50
60
Culture time (d)

Fig. 7

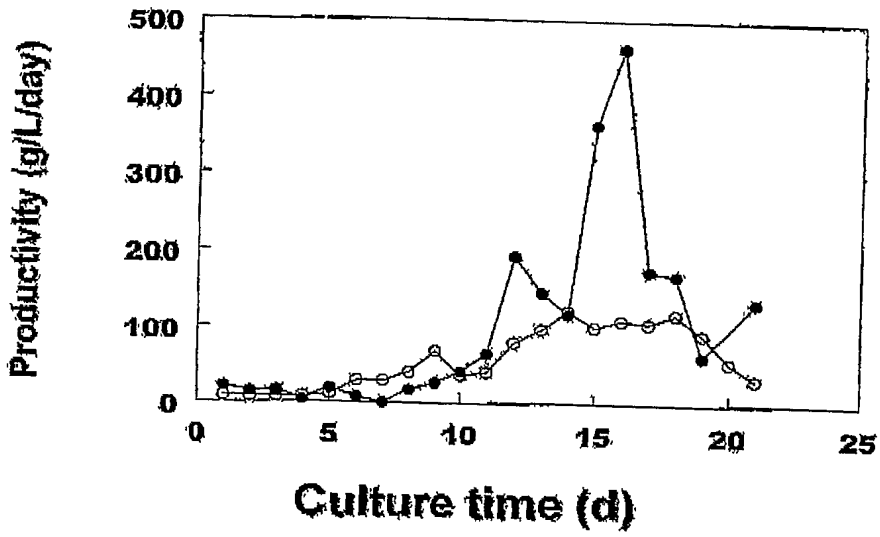

Productivity (g/L/day)
500
400
300
200
100
0
0
5
10
15
20
25
Culture time (d)

Fig. 8

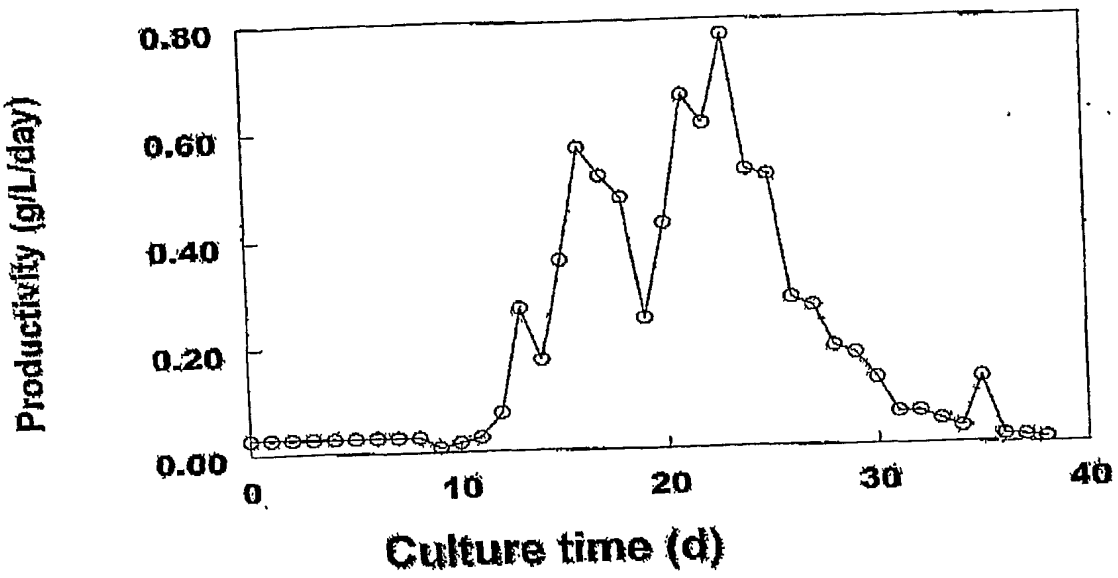

Productivity (g/L/day)
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
Culture time (d)

Fig. 9

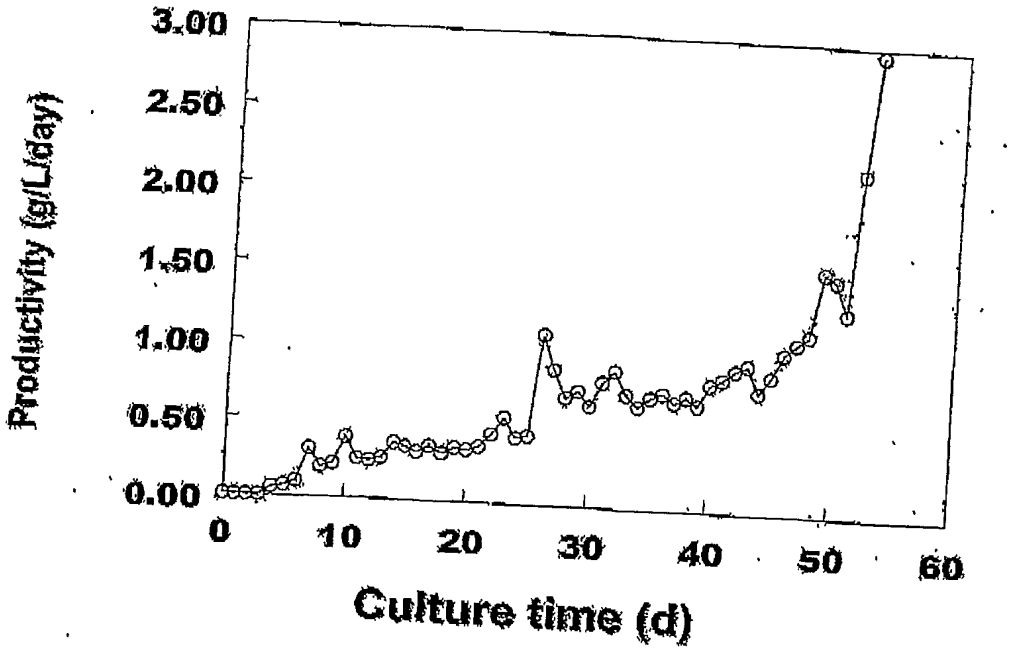

Productivity (g/L/day)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
Culture time (d)

Fig. 10

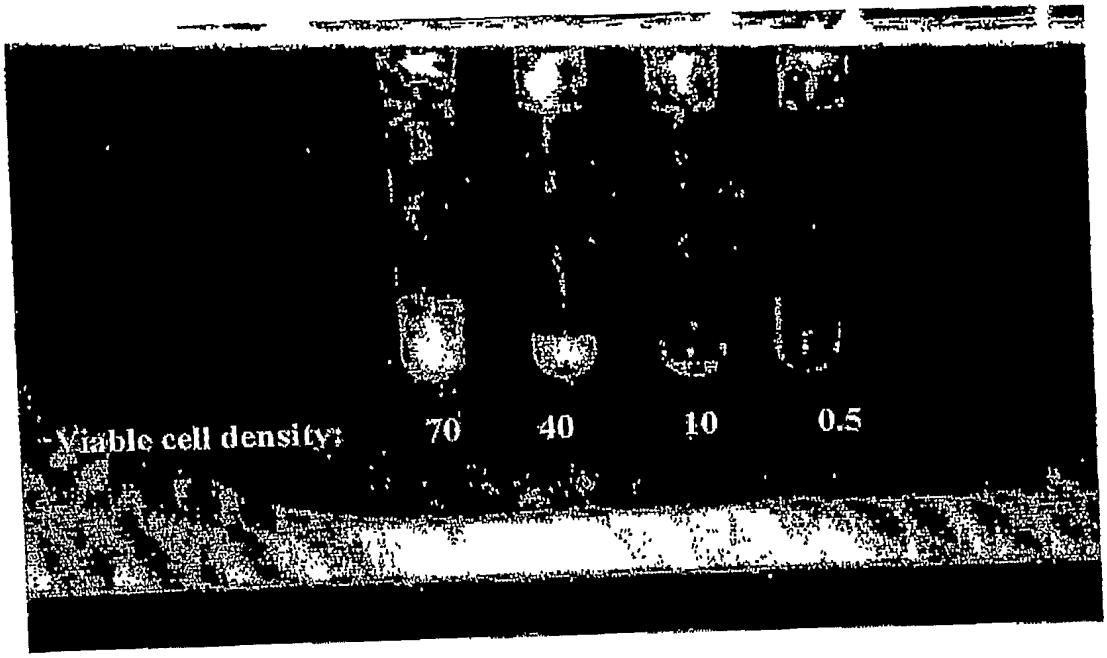
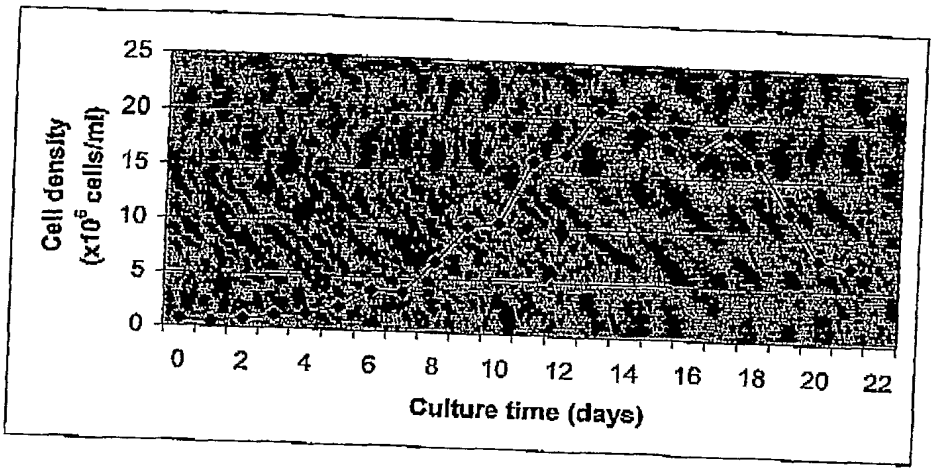


Fig. 11


Cell density
(x10^6 cells/ml)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20
22
Culture time (days)

5

Fig. 12

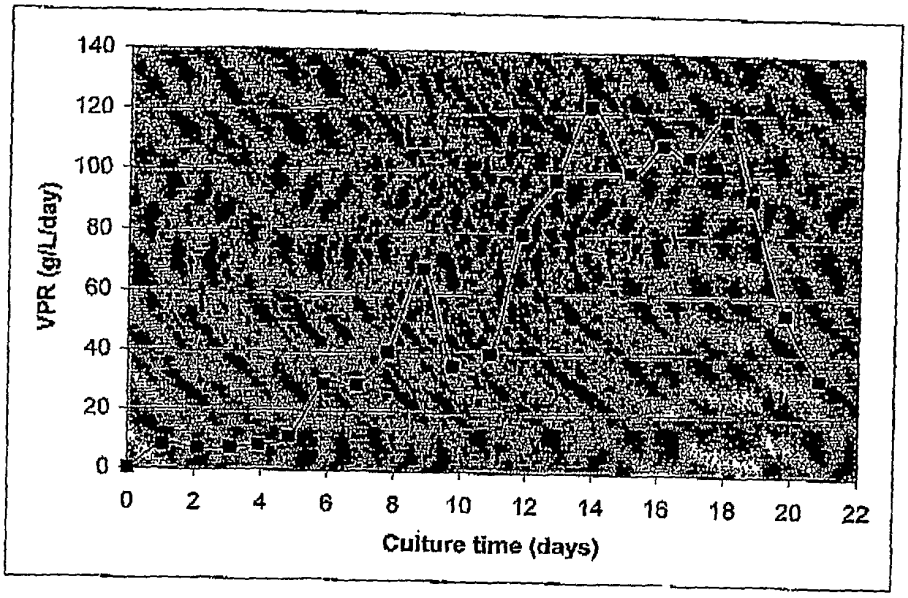

VPR (g/L/day)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
Culture time (days)

Fig. 13

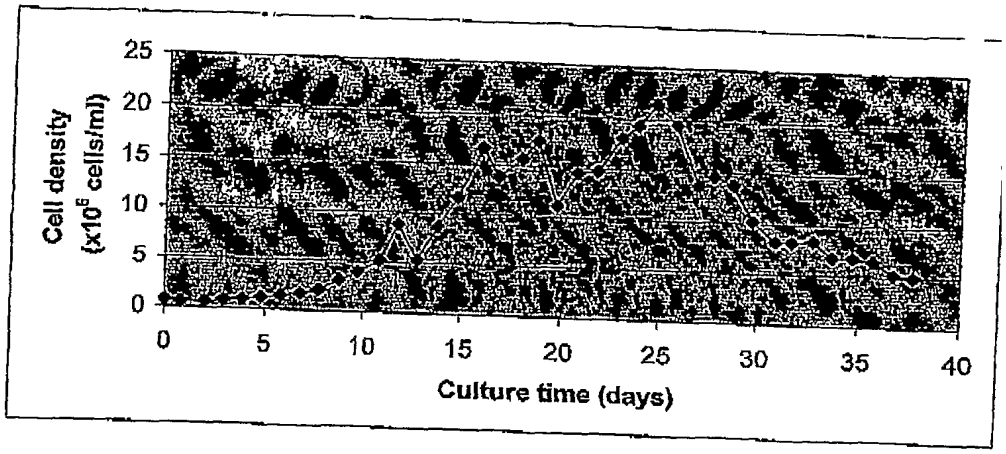
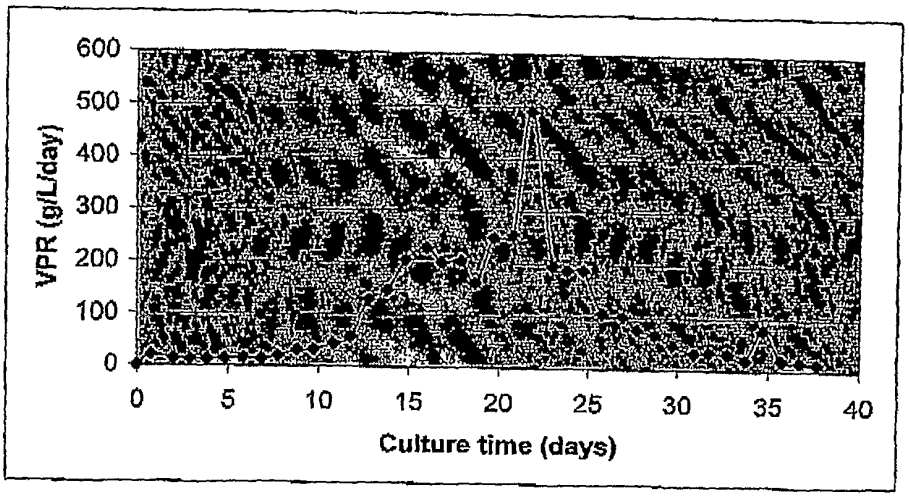

Cell density
(x10⁶ cells/ml)
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
Culture time (days)

Fig. 14


600
500
400
300
200
100
0
VPR (g/L/day)
0
5
10
15
20
25
30
35
40
Culture time (days)

5

Fig 15

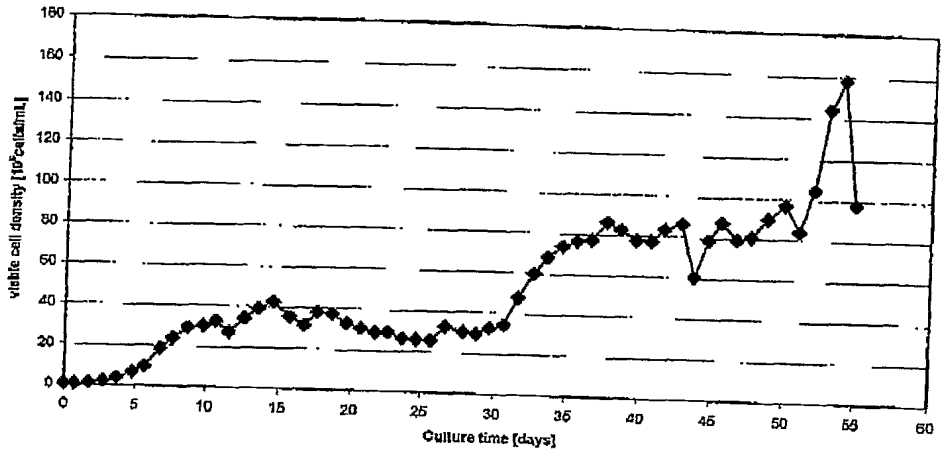
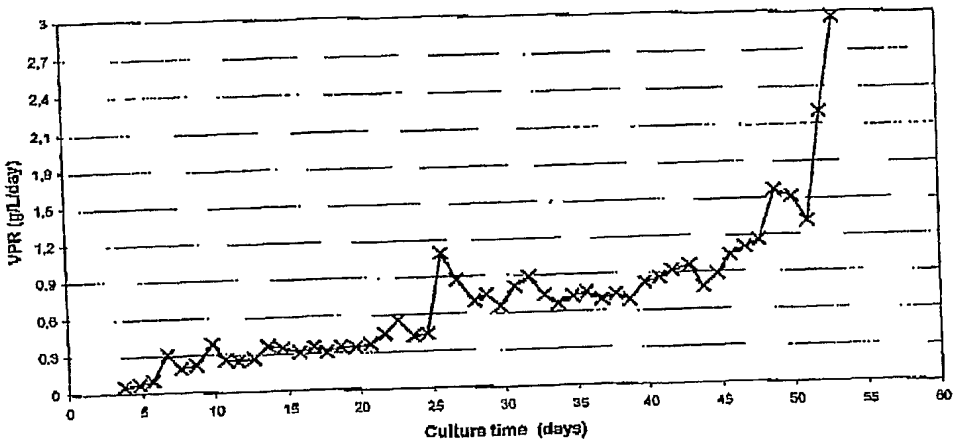

viable cell density [10⁵cell/ml]
Culture time [days]
0
20
40
60
80
100
120
140
160
180
0
5
10
15
20
25
30
35
40
45
50
55
60

Fig. 16


VPR (gr/L/day)
3
2,7
2,4
2,1
1,9
1,5
1,2
0,9
0,6
0,3
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Culture time (days)

5

Fig. 17

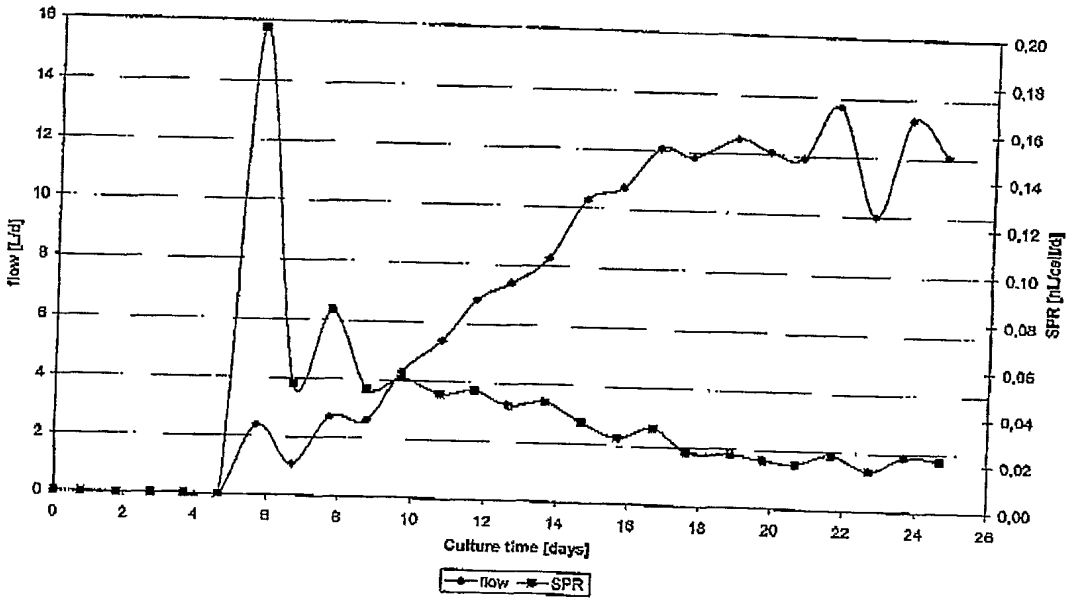
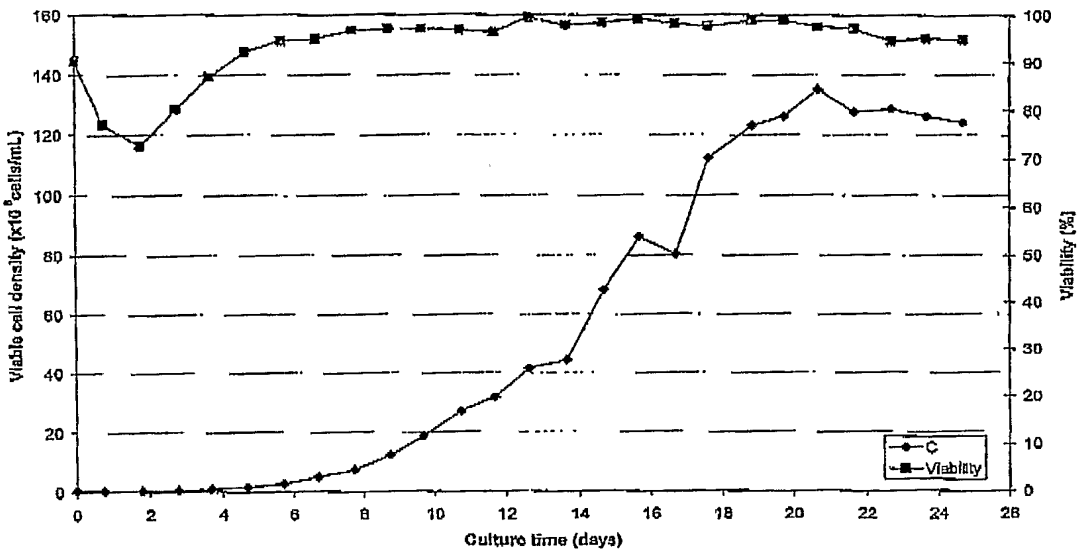

flow [L/d]
SFR [nL/cell/d]
Culture time [days]
flow
SPR
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0,00
0,02
0,04
0,06
0,08
0,10
0,12
0,14
0,16
0,18
0,20

Fig. 18


Viable cell density (x10 5 cells/mL)
Viability (%)
Culture time (days)
C
Viability
0
20
40
60
80
100
120
140
160
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14
16
18
20
22
24
28

5

10